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THERMOPHILIC BACTERIA

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THERMOPHILIC BACTERIA /*164

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I.

The interest of workers in recent years has repeatedly been attracted by the group of thermophilic bacteria.

The first data on bacterial growth at excessively high temperatures have been presented by Miquelher (Annuaires de l'observ. de Montsouris, 1881. — Les organismes viv de l'atm., 1883. — Monographie d'un bacille vivant au delà de 70°C., Annales de Micrograph., 1888, année I^{ère}, p. 4 - 10). Van Tieghem (Soc. bot. de France, Bull. 28) next described a streptococcus, which grew at 74°. Certes and Garrignon (Comptes rendus, 103, 703) were able to demonstrate small, motile as well as somewhat larger nonmotile rods in the waters of Luchon; the temperature of these hot spring lies at 64°. In general, all these cases—as Gotschlich has put it correctly—were more or less regarded as curiosities. Globig's investigations [Ueber Bacterienwachsthum bei 50 bis 70° (Bacterial growth at 50 to 70°), Zeitschrift für Hygiene, 3, 1888] have shown that there exist, in particular, in the upper layers of the earth, a relatively rich flora of micro-organisms. The latter author employed potato slices for the separation of the /165
various species since agar apparently was not suitable for that purpose. Globig's work was aimed chiefly at the elucidation of the natural conditions permitting growth of these bacteria at temperatures as high as that. In this regard, he ascribed an important role to the high temperatures brought about in the upper layers of the earth by insolation. Among the other contributions to our knowledge of the thermophilic bacteria, we mention F. Cohn (Berichte der deutschen botan. Gesellschaft, 1893, 66) and Macfadyen and Blaxall (Journ. of Paras. and Bact., 1894). The noteworthy result of the latter sutdies is found in the demonstration of the frequent appearance of these bacteria in the soil down to a depth of five feet, in river and lake water, in river sludge, and in the feces of man, mouse and chicken, respectively. In almost all studies, the authors were satisfied with the demonstration of the occurrence of thermophilic bacteria, while the more detailed characterization of the species was treated as a matter of secondary

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*Numbers in the margin indicate pagination in the foreign text.

importance. The studies undertaken by L. Rabinowitsch [Ueber die thermophilen Bacterien (The thermophilic bacteria), Zeitschrift für Hygiene, 20, 1895] were aimed at the latter point. Rabinowitsch has suggested that growth of thermophilic bacteria on agar and bouillon at low temperatures — approximately between 34 and 44° — is more marked under anaerobic than under aerobic conditions; in contrast, aerobic growth supposedly is more marked at higher temperatures. The wide distribution of thermophilic bacteria, already mentioned by certain other authors, was confirmed by L. Rabinowitsch.

These particular bacteria are represented by various species in the atmospheric dust; they can also be found in the intestinal tract of different warm- and cold-blooded animals as well as in that of man. The temperature limits for growth have also been determined; according to these earlier studies, all growth of the bacteria belonging to this group ceases at 75°. F. Cohn (Berichte der deutschen botanischen Gesellschaft, 1893) has ascribed to the thermophilic bacteria a role during the so-called self-heating of malt, tobacco leaves, cotton, hay, and dung; the latter suggestions agrees with the investigations carried out by Schlösing [Contribution à l'étude des fermentations du fumier (Contribution to the study of dung fermentation), Annales agronomiques, 18, 5, 1892]. /166

The characteristics known at the present of thermophilic bacteria are not adequate for the purpose of identification, and we must expect that the great number of investigations dealing with this subject may make a separation of the individual species more difficult. For that reason, I have made the attempt to determine the properties of a number of species paying particular attention to the questions of zymogenesis and enzymosis, which hitherto have been almost completely neglected.

For the isolation of the individual species we have followed a procedure frequently used in this Institute. The first step is aimed at obtaining accumulation of cells; for that purpose we have given the crude inoculation material into test tubes containing bouillon, which were then inoculated for 24 hours at 55°. We next prepared plates at different dilutions in Petri double dishes. Isolation was continued by repeated transfer inoculations on slanted 2% agar.

For primary inoculation we used soil samples from the Thiergarten, near Berlin, as well as Spree river water and Roquefort cheese; in another instance I used for that purpose blood serum, which had not become sterile following discontinuous sterilization carried out in the usual manner.

I next offer a detailed description of the species isolated in the course of my investigations:

The first species, which I have named Bacillus thermophilus liquefaciens aërobius because of its particular properties, was isolated from a soil sample. This bacterium is a rather slender rod exhibiting a tendency to form threads on various nutrient substartes. The spores usually are seen in the middle, and they do not exceed the diameter of the rods to any considerable extent.

On agar plates, this bacterium forms at 55°C uniform, finely granulated colonies exhibiting a yellow-brown center. Some of the flat colonies form delicate, pale gray beds with relatively irregular borders. Macroscopically the

majority of the colonies exhibit dentated borders or extending branches. Under the microscope, one sees thin, branched threads emanating in all directions from the borders. The colonies already after 24 hours reach a large size. On potato slices, these bacteria form well-developed beds already after 24 hours. The surface of these slices is covered with a dull, folded membrane; a membrane of that type can also be found on the surface of the condensed water. The potato slices acquire a reddish brown color. /167

On alkaline potato slices (prepared by dipping the slices for five minutes into a weak soda solution before sterilization) growth is poor at 60°; proliferation is almost visible, and it is of the same color as that of the potato slices. Involutional forms are numerous. Spores are present in small numbers. Development proceeds well at 55°, and spores appear only after 24 hours.

Growth on simple agar still takes place at 70°. A surface coat is formed regularly on bouillon; the bouillon becomes cloudy; sedimentation is minimal. Growth also is marked in alkaline peptone water. Growth is promoted when either 1% gelatin or 2% dextrose is added to the alkaline peptone water. In the latter case, the nutrient substrate becomes turbid, and some sediment appears. Milk is coagulated by these bacteria only after 48 hours.

On blood serum at 55° these colonies show a tendency to remain isolated. The blood serum becomes liquefied; sporulation takes place also on this substrate.

Growth on slanted agar takes place in the form of flat, well-developed layers; a thick membrane appears on the surface of the condensed water. Isolated colonies appear only rarely. On glycerinated agar, the coat formed is as rich as on the latter substrate; it is grayish-white and pasty. A thick, somewhat shagreened coat also forms on slanted dextrose agar. Despite the fact that I was dealing here with an exquisitely thermophilic bacterium, I did not disregard the advice given by Professor Dr. Günther viz. to determine the behavior of this bacterium also on gelatin. I proceeded by submerging the gelatin cultures grown at 55 - 60° in ice water. Since the liquid gelatin solidifies again during that procedure, one is able to determine whether the bacteria have exerted gelatinolytic action or not. That procedure has already been used by Bitter in connection with a different investigation [Ueber die Fermentausscheidung des Koch'schen Vibrio der Cholera asiatica (Enzyme excretion by Vibrio cholerae), Archiv f. Hyg., 5, 1886].

The gelatin-containing tubes were incubated at 55° following inoculation with the bacterium under consideration; growth was very marked already after 24 hours. A rich sediment had formed, and a distinct membrane had appeared on the surface. Using the procedure mentioned above, we were able to demonstrate liquefaction of the gelatin substrate neither after 24 nor after 48 hours of incubation, and the substrate again solidified fully in the ice water. At the same time, we had decided to study bacterial growth also on slanted gelatin at room temperature. In fact, we found that growth proceeded very slowly; distinct colonies appeared only four days after inoculation. These colonies formed slightly elevated disks with diameters of 2 to 3 mm. There were as yet no signs indicating liquefaction of the substrate. After eight days, however, we noted distinct liquefaction, which increased with time. /168

In gelatin stab cultures, liquefaction started on the surface, while the deeper colonies remained isolated for a relatively long period of time along the path of the inoculation stab. These deeper colonies required a period of ten days in order to develop their liquefying properties.

In 5% alkaline peptone gelatin substrate was liquefied by this bacterium in an infundibuliform manner at room temperature within six days. Only the upper half of the substrate was liquefied after 48 hours in a gelatin stab culture (simple nutrient gelatin) kept at 36° (tested using the ice water method). Increasing the temperature (to 41°) did not accelerate liquefaction. For that reason, we may assume as probable the existence of a temperature optimum for the development of the proteolytic properties of this particular bacterium. We have been unable so far to perform tests aimed at fixing this temperature optimum; however, it must be between 36 and 41°C.

This bacterium is able to grow also under hydrogen, but this markedly less rapidly and less well than under conditions with access of oxygen. Deprivation of oxygen also eliminates its ability to form spores. We have demonstrated this fact not only in the microscopic examination of the cultures, but also in tests concerned with their resistance against the effects of higher temperatures. We have, thus, found that a culture of this bacterium grown under hydrogen loses its ability to develop after heating to 85° for ten minutes.

Furthermore, the finding showing that cultures started in test tubes containing dextrose agar do not exhibit spore-carrying bacteria in the deeper layers can be interpreted as indicating that oxygen deficiency leads to the loss of the ability to form spores.

This bacterium is a nonmotile one. With respect to its reducing ability, we were able to determine only very weak reduction of litmus dye due to bacterial activity at 55°. When we grew the bacteria at 50°, we found that the litmus gelatin was liquefied, i.e. the dye turned reddish to a marked degree, and the blue color did not reappear despite repeated shaking. This finding indicates beyond doubt that this bacterium is an acid-forming one and a weakly reducing organism. Indole formation could be observed neither in simple peptone water nor in tap water; addition of potassium nitrite and sulfuric acid to the culture in simple bouillon did not bring about red coloration. We may mention that this culture was examined after 48 hours. Fermentation was observed neither in dextrose nor in lactose bouillon. /169

We were able to show pigment formation by this bacterium. A substrate particularly suitable for pigment formation demonstration is found in dextrose agar. When a thick layer of that substrate is used, the (reddish) dye appears directly on the surface and does not penetrate into the substrate.

The second species isolated by us has been given the name Bacillus thermophilus aërobius. We have isolated this bacterium from sewage in Berlin. It is characterized by the following growth and biological properties. It appears as a slender, nonmotile, slightly pointed bacterium; it readily stains when treated with the usual dye solutions. On glycerinated agar, this bacterium grows in long, slightly bent threads.

The bacteria form colonies on agar plates; these colonies spread on the surface reaching diameters between 7 and 10 mm after 24 hours. The colonies are bordered in a somewhat irregular, but sharp manner. They are very thin, veil-like, transparent and slightly iridescent. Under the microscope, these colonies exhibit uniform, fine granulation. No growth could be detected on either simple or alkaline potato slices.

Bouillon becomes turbid to a marked degree; the sediment film is sparsely developed; coat formation is never found on the surface.

Alkaline peptone water is just as good a substrate as bouillon. Milk is not coagulated by this bacterium. Addition of 2% dextrose or of 1% gelatin to the alkaline peptone water apparently does not promote growth to any definite extent. On blood serum, we noted delicate membranes; terminal spores were found also on that substrate. On slanted agar, we found formation of veil-like, transparent beds, and relatively large quantities of a dust-like sediment in the condensed water. Growth on this substrate was possible also at 35°, but it was less rapid and less extensive. The individual colonies remained isolated, and they did not cover the entire surface of the substrate.

On glycerinated agar, the bed was somewhat more distinct than on simple agar; on dextrose agar, bed formation was even more distinct. Addition of dextrose furthermore promoted sporulation. On simple agar, we were unable to demonstrate the presence of spores after a long period of growth; resistance against heating appears to point in the same direction viz. that no spores are formed on simple agar. For instance, a culture of that type tolerated only heating to 85° within five minutes without being killed; material used for transfer inoculation after heating to 85° for ten minutes did not yield growth. In simple bouillon, this bacterium formed alkali. Litmus gelatin was clearly discolored within 48 hours. Indole formation was not observed, and this also not after addition of potassium nitrite. Gas formation was observed in dextrose bouillon neither at 37° nor at 55°.

The third species to be described by us was isolated once from Spree river /170
water and once from the ice (of that river). With reference to the finding site, we have named this bacterium Bacillus thermophilus aquatilis. It is a thin, pointed bacterium with terminal spores.

On agar plates, this bacterium forms small, superficial, transparent and flat colonies having the appearance of dewdrops; these colonies exhibit a light brown tint. They are sharply bordered, finely grained, and they appear colorless under the microscope. Some small colonies growing within the medium exhibit whetstone shape. On simple potato slices, we observed a particularly well developed, yellowish brown, wet and uniform coating of smeary consistency.

On alkaline potato slices, the coat formed looks honey-like. On that substrate, rich growth took place also at 60°; spore formation at that temperature also is not inhibited. The potato slices acquired a marked brownish tint. In simple bouillon, that bacterium could not even grow following addition of dextrose. Alkaline peptone water, too, was not suitable for growth, and alkaline peptone water with added dextrose was just as unsuitable. Only following addition of 1% gelatin to alkaline peptone water did growth take place in this liquid substrate. In the latter, the bacterium formed numerous flakes and a rich sediment.

Milk was not coagulated. On blood serum, there appeared a wet, uniform coat; serum was not liquefied.

On slanted simple agar, the bacterium, starting from the inoculation scratch, formed an uniform, somewhat dull brownish coat, which finally covered the entire surface; the condensed water showed a rich sediment film but no covering membrane. The culture lost viability after 53 days. There was no growth at 35°, but rich growth was seen at 60°; however, at the latter temperature, the culture lost viability already after eight days. On glycerinated agar, we did not observe any particular growth characteristics. The bacterium did not grow on simple peptone gelatin at room temperature. At 55°, growth was marked; the surface of the substrate was covered by a coat.

Liquefaction did not take place at that temperature. No growth could be noted under hydrogen. The spores are terminal ones. The bacterium is nonmotile. Alkali was formed in simple bouillon. Litmus solution was slightly decolored. Indole was formed neither in peptone water nor in bouillon. Neither dextrose nor lactose was fermented by this bacterium.

The fourth species has been named Bacillus thermophilus reducens. We have isolated this bacterium from a blood serum-containing test tube which had not become sterile following the usual sterilization procedures.

On agar plates, this bacterium formed after 48 hours yellow to brown, uniform, finely granulated colonies with relatively sharp and regular borders. The somewhat convex-shaped colonies appeared dry and slightly shagreened. /171
No growth was seen on either simple or alkaline potato slices.

Bouillon became cloudy; a dust-like sediment film had formed after three days. Alkaline peptone water became turbid to a marked degree. Milk was not coagulated. Alkaline, dextrose-containing peptone water showed turbidity already after 24 hours; a coat was not formed on the surface of the substrate. The bacteria became somewhat larger, and they exhibited a slight bend.

Growth was poor on blood serum; the coat formed was hardly visible; sporulation, however, was marked.

On slanted simple agar there developed colonies, which usually remained isolated; a small band was formed along the streak. Development of the colonies was completed only after three days; these exhibited diameters maximally measuring 2 mm. No growth could be demonstrated on agar at either 60 or 35°. The condensed water was slightly turbid; it did not exhibit a sediment film. The colonies formed on glycerinated agar differed from those formed on agar to the extent that they appeared less dry. The colonies found dextrose agar appeared dull and dry, and they tended to spread more over the surface.

Rich growth was observed on simple gelatin after 48 hours; on that substrate, growth was still marked at 62°. No visible colonies developed at room temperature.

Rich growth was also seen on 5% alkaline peptone gelatin. No growth could be detected under hydrogen. The spores are terminal ones. This bacterium is an

alkali-former; it also is a strong reducer, and litmus solution was completely decolored at 55° already within 24 hours. Potassium nitrate, too, was reduced to some extent by this bacterium within three days. There was very weak indole formation. No fermentation took place in dextrose-containing bouillon.

We will now describe the fifth and final species, which we have named Bacillus thermophilus liquefaciens tyrogenus. This bacterium was isolated from Roquefort cheese. It is a more or less large, uniform and well staining bacterium with sharply truncated ends. On agar plates, this species formed small, round, dark brown and lobulate colonies which were finely granulated and have elevated centers. The center and the border area are somewhat lighter than the part in between. Growth ceased at 70°. On simple potato slices, dull, yellowish brown and folded membranes were formed already within 24 hours. Growth was moderate on alkaline potato slices; on that substrate, many involutional forms could already be seen after 24 hours; sporulation was small. The coat became wet with time and appeared smeary.

Bouillon became turbid, and a covering membrane could be seen. Growth was moderate on alkaline peptone water at 55°; spores nevertheless were formed; /172 there appeared a sparse, dusty sediment film. Growth was more marked in 1% gelatin-containing alkaline peptone water; the entire surface was covered by a membrane after 24 hours; there was a rich, flaky sediment. The liquid was turbid to a marked degree.

Sharply demarcated, slightly elevated, whitish colonies developed on blood serum. The serum was liquefied around the colonies. On slanted agar, growth was seen also at 35°. At +55°, there developed a rich, whitish, opaque coat; the sediment in the condensed water was scarce. Numerous branched furrows traversed the coat with time; the lower part of the coat was folded; its upper part was somewhat serrated. A folded, brownish red membrane covering the entire surface was seen on glycerinated agar; a number of isolated colonies exhibited dentated borders. Rich growth was also observed on dextrose agar.

Slow but distinct growth was seen on simple gelatin also at room temperature. The colonies measured 0.5 mm in diameter after three days; growth then continued so that the colonies measured 2 to 3 mm in diameter after ten days. Liquefaction started only after a certain period of time. Liquefaction took place earlier at higher temperatures. For instance, gelatin was completely liquefied after 48 hours at 41°; liquefaction decreased at still higher temperatures, and it ceased at 47°. No growth took place on gelatin at 60°. The bacterium grew well in an atmosphere of hydrogen, and sporulation did not cease.

Spores were found in the middle of the bacterium as well as at the ends. The bacterium is nonmotile. It forms acid in peptone water following addition of dextrose. The bacterium exhibits slight reducing ability. No gas formation was seen in dextrose bouillon. Indole could not be detected in either peptone water or bouillon, and this also not following addition of potassium nitrite.

We also observed crystal formation in a four-month-old culture on simple agar. Pigment formation took place in a relatively old culture on deep layers of dextrose agar.

I. Bacillus thermophilus liquefaciens aerophilus

1. Agar plates	Uniform, finely granulated colonies exhibiting a yellow brown tint in the center. The majority of the colonies exhibits dentated borders or branches. Under the microscope, one sees thin, apparently branched threads emanate from the borders into all directions.
2. Potato slices	Rich beds. The slices are covered with a dull, folded membrane. The coat is reddish brown in color.
3. Alkaline potato slices	Little growth at 60°. The growth is of the same color as that of the potato slice.
4. Bouillon	Cloudy turbidity with uniform coat formation. Slight sedimentation.
5. Alkaline peptone water	Rich growth.
6. Milk	Coagulated after 48 hours at 55°.
7. Alkaline peptone water with dextrose	Rich development.
8. Alkaline peptone water with 1% gelatin	Good growth.
9. Blood serum	The colonies are better isolated at 55°. Liquefaction takes place.
10. Slanted agar	Growth takes place still at 70°. At 55° there appeared a flat, grayish white, rich coat. The condensed water exhibited a membrane.
11. Glycerinated agar	The coat is as rich as that seen on agar; it is grayish white and pasty.
12. Dextrose agar	Thick, slightly shagreened coat.
13. Simple peptone gelatin	Rich growth at 55°. No liquefaction took place at that temperature. The gelatin was liquefied only below that temperature limit.
14. 5% alkaline peptone gelatin	Infundibuliform liquefaction occurred at 21° within six days.
15. Growth under hydrogen	Growth without spore formation.
16. Sporulation	The spores usually occupy the middle position.
17. Motility	Nonmotile.
18. Reducing ability	Potassium nitrate was not reduced.
19. Indole formation	None.
20. Fermentation	No gas formation in dextrose bouillon.
21. Locality	Soil from the Thiergarten.
22. Shape	Relatively slender bacterium with tendency to thread formation.
23. Pigment formation	A reddish pigment appeared on the surface of dextrose agar substrate.

II. *Bacillus thermophilus aerobius.*

1. Agar plates	The colonies spread widely across the surface. After 24 hours, the colonies measure 7 mm in diameter. They are somewhat irregularly, but sharply bordered. They are very thin, veil-like, transparent, and slightly iridescent. Under the microscope, the colonies appear uniformly and finely granulated.
2. Potato slices	No growth.
3. Alkaline potato slices	No growth.
4. Bouillon	Marked turbidity without coat formation.
5. Alkaline peptone water	Slight turbidity.
6. Milk	Not coagulated.
7. Alkaline peptone water with dextrose	The liquid was slightly turbid after 48 hours.
8. Alkaline peptone water with 1% gelatin	Slight turbidity after 48 hours.
9. Blood serum	Delicate coat formation, and spore formation.
10. Slanted agar	Growth evident also at 35°, but less rapid and less rich. The colonies remain isolated and do not cover the entire surface. Rich sediment present in the condensed water.
11. Glycerinated agar	The coat is somewhat better developed than on simple agar.
12. Dextrose agar	The coat is rich but less transparent than on simple agar. Only a superficial veil was formed on deep agar.
13. Simple gelatin	Rich development at 55°. The gelatin appears turbid due to the presence of a great number of flakes; rich sediment. Growth also at 60° (rich). Does not grow at room temperature. The gelatin is not liquefied.
14. 5% alkaline peptone gelatin	Rich growth.
15. Growth under hydrogen	No growth.
16. Sporulation	Terminal spores. Spore formation is promoted on dextrose agar.
17. Motility	Nonmotile.
18. Modification of the medium	Forms alkali.
19. Reducing ability	Markedly reducing after 48 hours.
20. Indole formation	No indole formation.
21. Fermentation	Does not cause fermentation at either 55° or 37°.
22. Locality	Sewage from Berlin.
23. Shape	Slender bacterium.
24. Pigment formation	None.

III. Bacillus thermophilus aquatilis

1. Agar plates	Macroscopically the agar surface appears to be covered with small dewdrops. The colonies are flat and exhibit a pale brown tint. They are sharply bordered, finely granulated, and almost colorless under the microscope. A few small colonies developing deep in the medium exhibit whetstone shape.
2. Potato slices	Particularly rich, yellowish brown, wet, uniform and slightly smeary coating.
3. Alkaline potato slices	Honey-like coating at 60°; rich spore formation at that temperature. The potato slices acquire a distinct, brown tint.
4. Bouillon	No growth.
5. Alkaline peptone water	No growth.
6. Milk	No coagulation.
7. Alkaline peptone water with 1% gelatin	Rich growth. The liquid does not appear to be cloudy; it contains a rich sediment of flakes.
8. Blood serum	Uniform, somewhat wet coating.
9. Slanted agar	No growth at 35°. Good defelopment at 60°. The culture grown at 60° can after eight days no longer be used for subsequent inoculation. At 55°, development of an uniform, thin coat, with rich sediment in the condensed water. The culture contained no viable cells after 53 days of growth.
10. Glycerinated agar	The coating is somewhat richer than the one developed on agar.
11. Slanted dextrose agar	Similar growth as on simple agar.
12. Simple peptone gelatin	No growth at room temperature.
13. Growth under hydrogen	No growth.
14. Sporulation	Terminal spores.
15. Motility	Nonmotile.
16. Modification of the medium	Forms alkali.
17. Reducing ability	Litmus solution gelatin is slightly decolored.
18. Indole formation	No indole formation.
19. Fermentation	The bacterium does not grow in dextrose bouillon.
20. Locality	Spree river water. Ice.
21. Shape	Thin, pointed bacterium.
22. Crystal formation	None.
23. Pigment formation	None.

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IV. Bacillus thermophilus reducens

1. Agar plates	Yellow to brown, uniformly and finely granulated colonies with relatively sharp, regular borders. The slightly convex-shaped colonies appear dry and somewhat shagreened.
2. Potato slices	No growth.
3. Alkaline potato slices	No growth.
4. Bouillon	The bouillon becomes cloudy with dust-like sediment.
5. Alkaline peptone water	Very turbid.
6. Milk	Not coagulated.
7. Alkaline peptone water with dextrose	The liquid becomes turbid at 55°. No membrane formation on the surface. The bacteria become a little longer, and they are sometimes bent.
8. Alkaline peptone water with 1% gelatin	Rich growth. Little but uniform turbidity. No coat formation. Little sediment.
9. Blood serum	Barely visible coat. Spores are formed.
10. Slanted agar	Colonies exhibiting the tendency to remain isolated. A narrow band is formed along the streak. Development is completed only after 3 days. The colonies do not measure more than 2 mm in diameter. No growth at 60°, and none either at 35°. The condensed water is slightly turbid. Little sediment formation.
11. Glycerinated agar	The colonies appear somewhat wet, and they are relatively viscid.
12. Dextrose agar	Relatively dry, somewhat dull coat.
13. Simple gelatin	Rich development after 48 hours, without liquefaction. Rich growth also at 62°. No growth at room temperature.
14. 5% alkaline peptone gelatin	Rich growth.
15. Growth under hydrogen	No growth on dextrose agar.
16. Sporulation	Terminal spores.
17. Motility	Nonmotile.
18. Modification of the medium	Forms alkali.
19. Reducing ability	Strongly reducing.
20. Indole formation	Red coloration appeared following addition of sulfuric acid to a simple, three-day-old bouillon culture to which potassium nitrite had been added.
21. Fermentation	The bacterium does not cause fermentation.
22. Locality	Obtained from a blood serum test tube.
23. Shape	Narrow bacterium.
24. Crystal formation	None.
25. Pigment formation	None.

V. Bacillus thermophilus

1. Agar plates	Small, round, dark brown, and finely granulated colonies with lobed, somewhat elevated centers. The center and the border are somewhat lighter than the other parts. Does not grow at 70°.
2. Potato slices	The surface of the potato slices is covered by a dull, yellowish brown and folded membrane.
3. Alkaline potato slices	Moderate growth after 24 hours. Many involuted forms. Few spores. The coat becomes wet and smeary with time.
4. Bouillon	Rich growth with membrane formation.
5. Alkaline peptone water	Moderate growth at 55°. Spores are being formed. Little, dust-like sediment.
6. Milk	Coagulation after 48 hours.
7. Alkaline peptone water with dextrose	Rich growth at 55°. The liquid becomes cloudy. No coat formation. Dust-like sediment.
8. Alkaline peptone water with 1% gelatin	The entire surface is covered by a membrane. The liquid becomes turbid. Rich, flaky sediment.
9. Blood serum	Well defined, somewhat elevated colonies.
10. Slanted agar	Growth also at 35°. Little sediment appears in the condensed water. Rich, opaque, whitish coat. Branched furrows appear with time. The coat is somewhat folded at the lower part of the streak, and somewhat dentated at the upper part.
11. Glycerinated agar	Reddish and folded membrane. A few isolated colonies exhibit serrated borders.
12. Dextrose agar	Rich growth.
13. Simple gelatin	Does not grow at 60°. Distinct growth is evident at room temperature. After 3 days, the colonies measure 0.5 mm in diameter. Liquefaction starts after ten days of growth. The gelatin is still being liquefied at 55°. Liquefaction ceases only at 57°.
14. 5% alkaline peptone gelatin	Rich growth.
15. Growth under hydrogen	Grows also under hydrogen. Spore formation is not affected.
16. Sporulation	Spores are seen in the middle as well as at the ends.
17. Motility	Nonmotile.
18. Modification of the medium	Forms acid in peptone water following addition of dextrose. The reaction is not changed on simple gelatin.
19. Reducing ability	Weakly reducing.
20. Indole formation	No indole formation.
21. Fermentation	Does not cause fermentation.
22. Locality	Isolated from Roquefort cheese.
23. Shape	Relatively slender bacterium, which frequently forms threads.
24. Pigment formation	In old cultures, a diffuse, reddish zone, which starts 5 mm below the surface, can be demonstrated in deep dextrose agar.

According to the results obtained in our studies, the group of thermophilic bacteria apparently exhibits very manifold differences with respect to their individual characteristics, and the apparent uniformity and similarity of their biological properties—indicated by certain earlier investigations—could not be confirmed by us. Not only did the purely morphological growth characteristics differ to a considerable extent, but the chemical processes of respiration and /178
consumption, enzymatic activity and tolerance for temperatures also proved to differ to a major degree, and these latter parameters differed among the few species studied by us not less than among species adjusted to lower temperatures.

The species isolated by me can only with difficulty be compared with those described by other authors since growth characteristics have been considered to a small extent only in the earlier studies. In the future, particular attention should be paid to the study of bacterial enzymatic activity.

The assertion made by L. Rabinowitsch viz. that the thermophilic bacteria are facultatively anaerobic organisms is not correct. We have observed species which did not grow at all in a carefully instituted oxygen-free atmosphere. In the thermophilic bacterium studied first by us, we cannot deny an effect of oxygen on sporulation.

II.

Once we had determined that two of the five species isolated by us exerted liquefying action on gelatin, we decided to study in some detail the peptonizing ability of these species. As valuable as the past studies on bacterial enzymes in general are, it is evident that it is well worth the effort to extend our knowledge also regarding the enzymes of the thermophilic bacteria. Before the presentation of my own experiments, it may be appropriate to review the literature dealing with the question on hand.

After Bienstock (Zeitschr. f. klin. Medic., 8, 1, 1884) had demonstrated that fibrin is transformed into propeptone (prepeptone) and peptone, respectively, by bacterial activities, Bitter (Arch. f. Hyg., 5, 1886) was able to show in /179
cholera vibriones that this peptonizing property must be attributed not directly to the living bacterial cell but to enzymes produced by that cell. Senger [Deutsche med. Wochenschr., No. 33/34, 1887, quoted after Flügge, Die Mikroorganismen (The microorganisms), 3rd edition, part 1, page 207] and Jerosch (quoted after Flügge) hold similar views. Rietsch and Sternberg, furthermore, have demonstrated "peptonizing enzymes" in Vibrio cholerae, Spirillium Finkler-Prior, Bac(terium) prodig(iosum), Pyocyaneus, and Staphylococcus pyogenes. Enzyme formation, however, became only in 1890 the subject of a detailed systematic study by Fermi ["Die Leim und Fibrin lösenden und die diastatischen Fermente" (The gelatin- and fibrin-digesting and the diastatic enzymes), Archiv f. Hyg., 10].

Credit is also due to the latter author for having developed a simple method for detecting enzymes in bacterial cultures. This method, which we have also used in our studies, requires the preparation of test tubes filled with 7% gelatin dissolved in saturated thymol water. It goes without saying that the "thymol gelatin" must be sterilized before use.

In a subsequent paper, the latter author has recommended the use of graduated test tubes for the quantitative estimation of the activity of the gelatinolytic agent. In addition, he has stressed the relatively greater sensitivity of gelatin compared to fibrin as reagent for tryptic enzymes, i.e. in cases where one is dealing with either a weak or a weakened tryptic enzyme, fibrin may not be suitable. In 1892, Fermi published a second study ["Weitere Untersuchungen über die tryptischen Enzyme der Mikroorganismen" (Further investigations on tryptic enzymes of microorganisms), Arch. f. Hyg., 14]. Since we take it for granted that the results of his studies are known to the reader, we believe there is no need to review them here; we shall mention his results only in instances where we have obtained different findings.

The study of the enzymatic properties of thermophilic bacteria is of particular interest since the high degrees of heat tolerated by these bacteria may bring about alterations in the activities of their enzymes. /180

We now present a number of experiments and their results, which point in the direction indicated above.

Experiment No. 1.

We have performed the following experiment in order to determine whether the activities exhibited by the two of our liquefying thermophilic bacteria can be ascribed to a chemical substance produced by them:

In each case we have used eight-day-old gelatin cultures grown at 41°. Five drops of the cultures were given into separate thymol gelatin test tubes. One percent carbolic acid had earlier been added to the gelatin cultures, and the thymol gelatin tubes used had been stored at 19°.

The results were as follows:

After eight days, the gelatin column in the tube,

in the case of Bacillus thermophilus liquefaciens aërobius, was liquefied to a level 5 mm below the surface and it could no longer be re-gelatinized, and

in the case of Bacillus thermophilus liquefaciens tyrogenus, was liquefied down to a level 3 mm below the surface and it could no longer be gelatinized.

Experiment No. 2.

Once formation of the tryptic enzyme in gelatin cultures had been ascertained in the experiment described above, the question arose whether bouillon would represent a substrate suitable for the production of this enzyme.

For this purpose, we used ten-day-old cultures grown at 41°. After passing the cultures through filter paper, we added 1% carbolic acid. Two ml. of the cultures treated in this manner were poured onto thymol gelatin.

The results are presented in the following Table:

	Thickness of the liquefied gelatin layer		
	1 day	After 3 days	1 week
Bacillus thermophilus liquefaciens aerobius	1.5 mm	4 mm	8 mm
Bacullus thermophilus liquefaciens tyrogenus	1 mm	3 mm	6 mm

That Table indicates that bouillon represents a substrate even more suitable for the formation of this enzyme than gelatin.

In order to determine whether enzyme formation takes place in bouillon also at 55°, we have performed the following experiment using Bacillus thermophilus liquefaciens aërobius.

Experiment No. 3. /181

A five-day-old culture grown at 55° was passed through double filter paper; 1% carbolic acid was added next. Two ml. of the culture treated in this manner was then given into a thymol gelatin tube. Inspection on day 5 of the test tube stored at 17° showed that a gelatin layer measuring 2 mm in thickness had been liquefied.

This result permits the conclusion that the temperature level of 55° is less favorable for enzyme formation.

Our next aim was to subject the activities of the enzymes produced by these two bacteria to a detailed study using fibrin.

Experiment No. 4.

In this experiment we used bouillon cultures grown at 41° and 20 days old. A few fibrin flakes, after careful washing in chloroform water, were added to 10 ml. of bouillon culture. In order to eliminate activity by the bacterial cells, we added alcoholic thymol solution to the liquid until it smelled strongly of thymol.

The Erlenmeyer flasks containing the liquid were incubated at 39°.

The results were as follows:

After eight hours of incubation, the Bacillus thermophilus liquefaciens aërobius culture exhibited distinct liquefaction of the fibrin flakes; after 12 hours, all the fibrin flakes added had been dissolved.

Liquefaction of fibrin flakes could also be seen after eight hours in the Bacillus thermophilus liquefaciens tyrogenus culture; that liquefaction, however, proceeded at a lesser rate than in the above mentioned culture.

The results obtained in the experiments outlined above lead to the conclusion that the enzymes produced by our two thermophilic bacteria possess significant proteolytic properties.

There still remained the task to examine these two species with respect to their amylolytic properties.

Experiment No. 5. /182

In order to obtain information regarding the possible existence of a diastatic enzyme in these two thermophilic species, we have mixed 10 ml. of the respective cultures with 5 ml. of starch paste using Erlenmeyer flasks. Activities by the bacterial cells were again excluded by the addition of thymol. The flasks were incubated at 37°. The cultures were tested for dextrose after 12 hours; the results, however, were negative.

Once the existence of proteolytic enzymes in the cases of the two isolated thermophilic species had been determined, we decided to make isolation of these enzymes our next task. However, I did not have the time to undertake the isolation of both enzymes, and we therefore selected Bacillus thermophilus liquefaciens aërobius for further study, since its enzyme had proved to be the more active one.

The continuation of my investigations in that direction was carried out in the Department of Chemistry of the Institute of Hygiene, under the direction of Dr. Winternitz, Assistant, to whom I am indebted for his friendly support of my work.

In the first experiment aimed at the separation of the enzyme from the other protein bodies present in the culture, we used a 20-day-old bouillon culture grown at 41°, which measured not more than 600 ml. That culture was first passed through double filter paper. Without concentrating the filtrate in vacuo, we precipitated it with the aid of alcohol. A ten-fold volume of culture was treated with 95% alcohol; filtration of the precipitate was carried out 24 hours later. Since we felt that a single precipitation would be inadequate for our purposes, we dissolved the precipitate obtained in 100 ml. of thymol water, and repeated the precipitation procedure using 80% alcohol. The precipitation took somewhat longer; the liquid obtained had an opalescent appearance. After 24 hours, we separated the precipitate from the liquid by filtration. The precipitate was removed from the filter and dried, and then dissolved in 100 ml. of thymol water. The liquid did not give /183 the Biuret reaction. Due to lack of time, I was forced to forgo removal of the salts from the enzyme solution by means of dialysis.

We next tested the liquid obtained with respect to its enzymatic properties. For that purpose we performed the following experiments:

a) Proteolytic enzyme assay

Five ml. of the liquid obtained were mixed with an equal quantity of thymol water and a few small flakes of fibrin; this mixture was incubated at 39°. Liquefaction of the fibrin flakes was evident after 5 hours; the flakes had completely disappeared after 12 hours. A specimen of that liquid gave a strong Biuret reaction.

b) Diastatic enzyme assay

Five ml. of the enzyme solution were mixed with 10 ml. of starch paste and a little thymol; this mixture was incubated at 39°. After 12 hours, we obtained marked reduction in Trommer's test.

These two experimental assays in an unequivocal manner demonstrate the existence of a proteolytic and of a diastatic enzyme in our material. We next wished to determine the temperature at which the proteolytic and the diastatic enzyme, respectively, loses activity.

For that purpose we have heated 5 ml. specimens of the enzyme solution in test tubes for one hour at 65 and 60°, respectively, and have then tested the solutions with respect to their activities on fibrin.

The results of these tests showed that the activity of the proteolytic enzyme in the solution was lost between 60 and 65°.

With respect to the resistance of the diastatic enzyme, repeated tests demonstrated that its resistance against heating is far greater. For instance, heating for one hour at 65° did not affect this enzyme. In other tests, we have subjected the enzyme to heating for one hour at 80°, and found that this temperature, too, did not affect the activity of this enzyme.

We performed the following experiment in order to determine exactly the /184
temperature limit at which the activity of this diastatic enzyme is lost.

Five ml. of the enzyme solution were given into a test tube sitting in a water bath. The test solution was subjected to a constant temperature of exactly 85° for 30 minutes. During the entire test, the thermometer was at all times kept in the middle of the solution. The enzyme solution pretreated in this manner was then mixed with 5 ml. of starch paste and incubated at 39°.

The Trommer's test, carried out after 20 hours, gave distinct reduction indicating that this diastatic enzyme tolerates heating at 85° for 30 minutes.

An additional test demonstrated that heating for 30 minutes at 88° destroys this enzyme.

The results of these tests on first approximation then appear to show that this enzyme possesses a greater resistance against heating than the enzymes hitherto known. Until now, it has been believed according to Hammarsten ["Lehrbuch der physiologischen Chemie" (Textbook on biochemistry), 1891, page 8] that heating to 80° will completely destroy enzymes.

Since Biernacki (Zeitschrift für Biologie, New Series, 10, 1891), however, has demonstrated that admixtures of protein bodies or of salts in the enzyme solutions are able both to protect enzymes against the consequences of heating and to increase the degree of heat required to destroy them, one could ascribe the greater resistance of our enzymes to that factor, since our enzyme solutions had not been purified in order to remove the salts present.

Additional experiments have shown that a neutral or weakly alkaline reaction is required to obtain optimal development of the proteolytic properties of this enzyme. In this respect, our enzyme resembles trypsin. In the presence of hydrochloric acid the enzyme was inactive on fibrin. /185

Since we had obtained a strongly proteolytic bacterial enzyme, we felt it was imperative to determine whether this enzyme was able to transform fibrin into peptone (as postulated by Kuhne). The reason for this undertaking was given in the view recently voiced by Fermi viz. that proteolytic enzymes bring about only liquefaction but not peptonization of protein compounds [Cl. Fermi, Se i microorganismi peptonizone l'albumine (Microorganisms peptonizing proteins), Centralblatt fur Bacteriologie, 1896, page 387]. For our purpose we have mixed 20 ml. of the enzyme solution with 30 ml. of thymol water, and have then permitted that mixture to act upon a relatively large quantity of fibrin. We must mention here the fact that the enzyme solution did not give a positive Biuret reaction. Following removal of the undigested fibrin flakes from the solution by filtration, we added excess ammonium sulfate to the solution with heating. After cooling, we separated the precipitated albumoses from the solution by filtration on the next day. The ammonium sulfate present in the solution was completely removed by adding barium hydroxide with heating. The latter compound was precipitated with the aid of diluted sulfuric acid. The liquid obtained in this manner was then concentrated to a volume of 10 ml. Nessler's reagent showed the presence of only minimal traces of ammonia.

In that solution we obtained a clearly positive Biuret reaction indicating the presence of peptone.

On the basis of this test, which, to be sure, was performed only once, we are thus permitted to assume that peptone arose from fibrin under the influence of this bacterial enzyme; the view held by Fermi, thus, does not have general applicability.

The result obtained by us, however, is not an entirely isolated one in this field of work. Kuhne has ascribed peptonizing activities even to the tubercle bacillus and the hay bacillus (i.e. Bac. subtilis; Translator) [Kühne, "Erfahrungen uber Albumose and Peptone (Experience on albumoses and peptones), Zeitschrift für Biologie, New Series, 11, 1892]. /186

In particular we believe we should stress the effects of heating on liquefactability, which the bacteria described loss at higher temperatures. We furthermore wish to draw attention to the fact that one, in cases where diastatic activities are to be determined, must obtain the pure enzymes from the cultures, since these enzymes can be diluted in the original cultures to an extent that they are unable to show activity; that was the case in the Bacillus thermophilus liquefaciens studied by us.